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The adenosine receptor affinities and monoamine oxidase B inhibitory properties of sulfanylphthalimide analogues

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ABSTRACT

Based on a report that sulfanylphthalimides are highly potent monoamine oxidase (MAO) B selective inhibitors, the present study examines the adenosine receptor affinities and MAO-B inhibitory properties of a series of 4- and 5-sulfanylphthalimide analogues. Since adenosine antagonists (A_1 and A_{2A} subtypes) and MAO-B inhibitors are considered agents for the therapy of neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease, dual-target-directed drugs that antagonize adenosine receptors and inhibit MAO-B may have enhanced therapeutic value. The results document that the sulfanylphthalimide analogues are selective for the adenosine A_1 receptor over the A_{2A} receptor subtype, with a number of compounds also possessing MAO-B inhibitory properties. Among the compounds evaluated, 5-[(4-methoxybenzyl)sulfanyl]phthalimide was found to possess the highest binding affinity to adenosine A_1 receptors with a K_i value of 0.369 μM. This compound is reported to also inhibit MAO-B with an IC_{50} value of 0.020 μM. Such dual-target-directed compounds may act synergistic in the treatment of Parkinson's disease: antagonism of the A_1 receptor may facilitate dopamine release, while MAO-B inhibition may reduce dopamine metabolism. Additionally, dual-target-directed compounds may find therapeutic value in Alzheimer's disease: antagonism of the A_1 receptor may be beneficial in the treatment of cognitive dysfunction, while MAO-B inhibition may exhibit neuroprotective properties. In neurological diseases, such as Parkinson's disease and Alzheimer's disease, dual-target-directed drugs are expected to be advantageous over single-target treatments.

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder, characterized pathologically by the loss of dopaminergic nigrostriatal neurons and clinically by disabling movement disorders [1]. The current therapy of PD relies mainly on dopamine replacement strategies with L-DOPA, the metabolic precursor of dopamine, and dopamine agonist drugs as the most useful agents [2]. Although these strategies are highly effective in controlling the early stages of the disease, long-term treatment is associated with drug-related complications such as loss of drug efficacy, the onset of dyskinesia and the occurrence of psychosis and depression [2]. The inadequacies of dopamine replacement therapy emphasise the need for more effective drugs for the treatment of PD. One non-dopaminergic approach that has been explored, is the blockade of adenosine receptors [3]. Four subtypes of adenosine receptors have been identified in the central nervous system (CNS), the A_1, A_{2A}, A_{2B} and A_3 subtypes, of which adenosine A_1 and A_{2A} receptors represent targets for the development of PD therapies [4]. Adenosine A_{2A} receptors are mainly localized in the striatum where A_{2A} receptor activation opposes dopamine D_2 receptor signalling [4,5]. Adenosine A_{2A} antagonists, in turn, have been shown to facilitate dopamine receptor signalling and thereby restore motor function in animal models of Parkinson's disease [3]. In this respect, adenosine A_{2A} antagonists potentiate the motor benefit of L-DOPA in Parkinson's disease patients, without the potentiation of L-DOPA-associated dyskinesia [6]. In contrast to adenosine A_{2A} receptors, adenosine A_1 receptors are widely expressed in the CNS. In the striatum, adenosine A_1 receptor stimulation inhibits dopamine release [7]. Conversely, blockade of A_1 receptors facilitates dopamine release in the striatum and potentiates dopamine mediated responses [7]. Additionally, it has been reported that adenosine

Abbreviations: PD, Parkinson's disease; AD, Alzheimer's disease; CNS, central nervous system; MAO, monoamine oxidase; [^{3}H]DPCPX, [^{3}H]-dipropyl-8-cyclopentylxanthine; [^{3}H]NECA, N-[^{3}H]-ethyladenosin-5′-uronamide; CPA, N6-cyclopentyladenosine; DMPX, 3,7-dimethyl-1-propargylxanthine; SAR, Structure–activity relationship; SEM, standard error of the mean; SD, standard deviation.

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A_1 receptor antagonists may improve learning and memory in animal models [7]. Adenosine A_1 receptor antagonists may thus also find therapeutic application by enhancing cognitive function in PD.

Furthermore, adenosine A_1 and A_{2A} receptors may also find therapeutic value in Alzheimer's disease (AD), another well-known neurological disorder. Pathologically, AD may be characterized by a marked loss of hippocampal and cortical neurons that clinically produces an impairment of memory and cognitive ability [8]. Selective adenosine A_1 receptor antagonists have been indicated to possess therapeutic value in the treatment of AD's cognitive dysfunction [9]. This may be attributed to the CNS stimulatory effects associated with adenosine A_1 receptor antagonists [9]. Adenosine A_{2A} receptor antagonists show promise in the treatment of AD, due to their neuroprotective effects observed during preclinical studies [9,10].

Based on the above considerations, the discovery of new adenosine receptor antagonists is pursued by several research groups. Of particular interest are reports that certain adenosine receptor antagonists, such as xanthine and benzothiazinone derivatives, also act as inhibitors of the enzyme monoamine oxidase (MAO) B [11,12]. For example, (*E*)-8-(3-chlorostyryl)caffeine (CSC, **1**; K_i = 26–54 nM) [13–15], a frequently used reference A_{2A} antagonist, also is a highly potent MAO-B inhibitor with a K_i value of 80.6 nM (Fig. 1) [11,16]. In the CNS, MAO-B represents a major catabolic enzyme of dopamine, and inhibitors of MAO-B have been used in PD to conserve depleted dopamine reserves [17]. MAO-B inhibitors are thus established therapy in PD and are often used in combination with L-DOPA, or as mono-therapy in early PD [17]. There is also evidence that MAO-B inhibition protect against neurodegeneration in PD, presumably by reducing the formation of neurotoxic aldehyde species and hydrogen peroxide, which are by-products of the MAO catalytic cycle [18]. Additionally, the neuroprotective properties associated with MAO-B may also be beneficial for AD treatment [9].

Dual-target-directed compounds that antagonize adenosine receptors and inhibit MAO-B may thus act synergistic in the treatment of PD: antagonism of the A_1 and A_{2A} receptors may facilitate dopamine release and signalling, while MAO-B inhibition may reduce the central catabolism of dopamine. Therefore, such compounds may have enhanced therapeutic value in PD. Additionally, it may be reasoned that dual-target directed compounds may find therapeutic value in the treatment of AD: antagonism of the A_1 receptor may be beneficial in the treatment of cognitive dysfunction, while antagonism of the A_{2A} receptor and MAO-B inhibition may exhibit neuroprotective effects. In neurological diseases, such as PD and AD, dual-target-directed therapeutics is expected to be advantageous over single-target treatments [9,10].

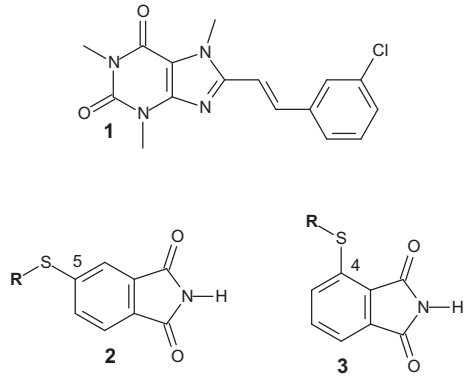


Fig. 1. The structure of CSC (**1**) and the general structures of 5-sulfanylphthalimide analogues (**2**) and 4-sulfanylphthalimide analogues (**3**) are also shown.

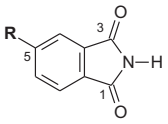
In the current study we have attempted to discover additional dual-target-directed compounds that antagonize adenosine receptors and inhibit MAO-B. A recent report has shown that a series of 5-sulfanylphthalimide analogues (**2**) are highly potent inhibitors of MAO-B [19]. Certain 5-sulfanylphthalimides were also potent inhibitors of the MAO-A isoform. For example, 5-[(4-bromobenzyl)sulfanyl]phthalimide (**2h**) inhibits human MAO-A and MAO-B with IC_{50} values of 0.273 μM and 0.0074 μM, respectively. MAO-A inhibitors are clinically used to treat depressive illness, and since a significant proportion of PD patients also exhibit signs of depression, the MAO-A inhibitory activities of these homologues may be beneficial in PD therapy [18]. Based on the favourable MAO inhibition profile of the reported 5-sulfanylphthalimides, the current study examined the possibility that these compounds also may possess affinities for adenosine receptors. For this purpose, the affinities of 9 of the reported 5-sulfanylphthalimides for rat adenosine A_1 and A_{2A} receptors were evaluated. To sample chemical space, phthalimides containing the phenylsulfanyl (**2a**), benzylsulfanyl (**2b**) and phenylethylsulfanyl (**2c**) substituents on C5 were selected (Table 1). 5-(Phenylsulfanyl)- and 5-(benzylsulfanyl)phthalimide were further substituted on the phenyl and benzyl rings with halogens (F, Cl and Br) and an alkyl (OCH_3) group to yield **2d**–**i**. We have further extended the series of sulfanylphthalimides by synthesizing a homologous series of 4-sulfanylphthalimides, compounds **3a**–**i**. The affinities of these homologues for rat adenosine A_1 and A_{2A} receptors, as well as their inhibition potencies towards human MAO-A and MAO-B were subsequently measured and compared to those of the 5-sulfanylphthalimides.

2. Results and discussion

2.1. Chemistry

The 5-sulfanylphthalimide analogues were synthesized as previously reported [19]. The 4-sulfanylphthalimide analogues (**3a**–**i**) were synthesized according to a similar protocol by reacting an appropriate thiol with 3-nitrophthalimide in the presence of K_2CO_3 (Fig. 2). Acetone served as solvent for these reactions. The target compounds were obtained with moderate to high yields (30–70%), and were purified by crystallization as cited in the

Table 1
The K_i values for the binding of 5-sulfanylphthalimide analogues **2** to rat adenosine A_1 and A_{2A} receptors, and the reported IC_{50} values for the inhibition of human MAO-A and MAO-B.



		K_i (μM)[a]		IC_{50} (μM)[b]	
	R	A_1	A_{2A}	MAO-A	MAO-B
2a	C_6H_5S–	0.648 ± 0.11	No affinity[c]	8.03	0.986
2b	$C_6H_5CH_2S$–	0.595 ± 0.04	No affinity[c]	1.92	0.0045
2c	$C_6H_5(CH_2)_2S$–	1.88 ± 0.32	No affinity[c]	2.27	0.030
2d	(4-ClC_6H_4)S–	No affinity[c]	No affinity[c]	1.68	0.457
2e	(4-BrC_6H_4)S–	No affinity[c]	No affinity[c]	1.01	0.364
2f	(4-FC_6H_4)CH_2S–	36.7 ± 1.19	No affinity[c]	0.958	0.0068
2g	(4-ClC_6H_4)CH_2S–	No affinity[c]	No affinity[c]	0.506	0.0056
2h	(4-BrC_6H_4)CH_2S–	0.676 ± 0.19	No affinity[c]	0.273	0.0074
2i	(4-$OCH_3C_6H_4$)CH_2S–	0.369 ± 0.02	No affinity[c]	1.63	0.020

[a] Values are expressed as the mean ± standard error of the mean (SEM) of duplicate determinations.
[b] Values obtained from reference [19].
[c] No affinity observed at a maximum concentration of 100 μM of the test compound.

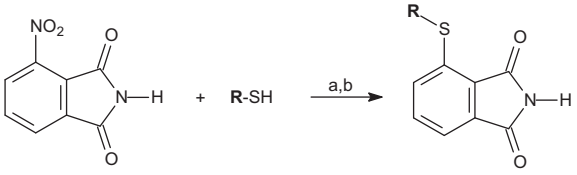


Fig. 2. Synthetic pathway to 4-sulfanylphthalimide analogues (**3a–i**). Reagents and conditions: (a) K_2CO_3, acetone, reflux, 24 h; (b) HCl (6 N).

supplementary. In each instance, the structures of the target compounds were verified by ^{1}H NMR, ^{13}C NMR and mass spectrometry (MS). The purities of the test compounds were evaluated by high performance liquid chromatography (HPLC) as cited in the supplementary. The molecular structure of **3i** was elucidated by X-ray crystallography and confirms substitution on the C4 position of the phthalimide moiety (Fig. 3).

2.2. In vitro binding to A_1 and A_{2A} receptors

The affinities of the sulfanylphthalimide analogues for rat adenosine A_1 and A_{2A} receptors were evaluated by the radioligand binding protocol described in literature [15,20]. The binding of the test compounds to adenosine A_1 receptors were evaluated by measuring the displacement of 1,3-[^{3}H]-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX) from rat whole brain membranes (Fig. 4). Binding of the test compounds to adenosine A_{2A} receptor, in turn, was evaluated by measuring the displacement of N-[^{3}H]ethyladenosin-5′-uronamide ([^{3}H]NECA) from rat striatal membranes. The adenosine A_{2A} receptor *in vitro* binding studies were carried out in the presence of N^6-cyclopentyladenosine (CPA) to minimize the binding of [^{3}H]NECA to A_1 receptors. The results of these binding studies are given in Tables 1 and 2. The results show that among the sulfanylphthalimide analogues (**2** and **3**) evaluated, 11 compounds possess affinity for adenosine A_1 receptors while 3 homologues exhibit affinity for A_{2A} receptors. It is noteworthy that none of the 5-sulfanylphthalimide analogues (**2**) possess affinity for A_{2A} receptors at a maximal tested concentration of 100 μM, while compounds among both the 5-sulfanylphthalimide (**2**) and 4-sulfanylphthalimide (**3**) analogues were found to bind to A_1 receptors. Among the compounds evaluated, compound **2i** was found to possess the highest binding affinity to adenosine A_1 receptors with a K_i value of 0.369 μM. Another noteworthy compound is **3b**, which exhibits the second highest binding affinity to adenosine A_1 receptors (K_i = 0.548 μM). This compound also possesses affinity for adenosine A_{2A} receptors with a K_i value of 0.980 μM. Among the series of sulfanylphthalimides, **3b** thus possesses the highest binding affinity to adenosine A_{2A} receptors and represents a compound with dual affinities for A_1 and A_{2A} receptors. It should be noted that although the affinities for adenosine receptors recorded here are relatively low, it is comparable to first generation antagonists such as 3,7-dimethyl-1-propargylxanthine

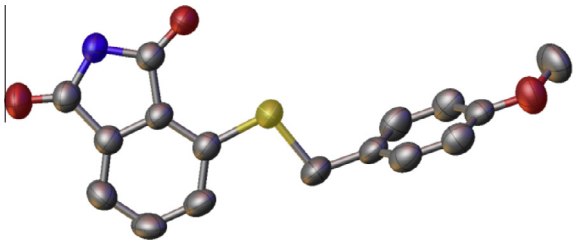


Fig. 3. The X-ray crystallographic structure of **3i** shown with displacement ellipsoids.

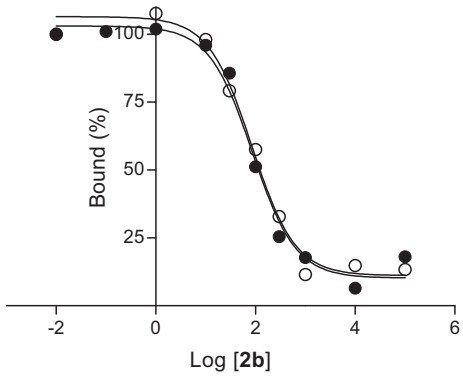


Fig. 4. The replicate sigmoidal dose–response curves for the binding of **2b** (expressed in nM) to rat A_1 receptors. The bound cpm values were adjusted for nonspecific binding and are expressed as percentage of the cpm values recorded in the absence of **2b**.

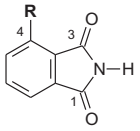
(DMPX) which exhibit K_i values for binding to A_1 and A_{2A} receptors of 12 μM and 8.6 μM, respectively [14]. While no clear structure–activity relationships (SARs) are apparent, it may be concluded that the sulfanylphthalimide analogues possess higher affinities for adenosine A_1 receptors than A_{2A} receptors since, for those homologues which exhibit affinities for A_1 receptors, the binding affinities at A_1 receptors are superior to those recorded at A_{2A} receptors.

2.3. In vitro inhibition of MAO-B

In order to evaluate the possibility that the sulfanylphthalimide analogues may act as dual-target-directed compounds, the 4-sulfanylphthalimide analogues (**3a–i**) were examined as inhibitors of recombinant human MAO-A and MAO-B. The human MAO inhibitory properties of the 5-sulfanylphthalimide analogues (**2a–i**) have been previously reported [19]. The enzymatic activities were determined fluorometrically by measuring the extent to which the mixed MAO-A/B substrate, kynuramine, is oxidized by the MAOs [21,22]. Kynuramine is oxidized by MAO-A and MAO-B to yield 4-hydroxyquinoline, a fluorescent metabolite (λ_{ex} = 310 nm; λ_{em} = 400 nm). Under the experimental conditions, neither kynuramine nor the test inhibitors exhibit fluorescence. The MAO inhibition potencies of the sulfanylphthalimide analogues are expressed as the corresponding IC_{50} values, and are given in Tables 1 and 2.

The results show that, although none of the 4-sulfanylphthalimide analogues (**3a–i**) were MAO-A inhibitors (up to a maximal concentration of 100 μM), most homologues (7 of 9) were MAO-B inhibitors. The most potent MAO-B inhibitor, compound **3f** exhibited an IC_{50} value of 0.290 μM. Other notable MAO-B inhibitors with submicromolar IC_{50} values were **3g** (IC_{50} = 0.460 μM) and **3e** (IC_{50} = 0.443 μM). These MAO-B inhibition potencies are, however, modest compared to the dual-target-directed compound, CSC, which inhibits MAO-B with an IC_{50} value of 0.146 μM [23]. Compared to the MAO inhibitory properties of the previously reported 5-sulfanylphthalimide analogues (**2a–i**), the 4-sulfanylphthalimide analogues are also modest inhibitors. For example, the 5-sulfanylphthalimide analogues (**2a–i**) are reported to all inhibit human MAO-A with 3 homologues exhibiting IC_{50} values in the submicromolar range. The 5-sulfanylphthalimide analogues are also in each instance more potent MAO-B inhibitors than the 4-substituted homologues with all IC_{50} values in the submicromolar range. In this respect, compound **2i** is particularly noteworthy since it not only represents a highly potent MAO-B inhibitor (IC_{50} = 0.020 μM), but also exhibits the highest affinity for the adenosine A_1 receptor (K_i = 0.369 μM) among the compounds evaluated. This compound may thus be considered as the most

Table 2
The K_i values for the binding of 4-sulfanylphthalimide analogues **3** to rat adenosine A_1 and A_{2A} receptors, and the experimental IC_{50} values for the inhibition of human MAO-A and MAO-B.



		K_i (μM)[a]		IC_{50} (μM)[b]	
	R	A_1	A_{2A}	MAO-A	MAO-B
3a	C_6H_5S–	7.72 ± 1.95	No affinity[c]	No inhibition[c]	9.17 ± 0.58
3b	$C_6H_5CH_2S$–	0.548 ± 0.03	0.980 ± 0.15	No inhibition[c]	1.88 ± 1.52
3c	$C_6H_5(CH_2)_2S$–	0.649 ± 0.02	17.0 ± 0.30	No inhibition[c]	3.90 ± 1.77
3d	(4-ClC_6H_4)S–	No affinity[c]	No affinity[c]	No inhibition[c]	1.39 ± 0.50
3e	(4-BrC_6H_4)S–	No affinity[c]	No affinity[c]	No inhibition[c]	0.443 ± 0.14
3f	(4-FC_6H_4)CH_2S–	6.30 ± 0.28	12.7 ± 0.09	No inhibition[c]	0.290 ± 0.07
3g	(4-ClC_6H_4)CH_2S–	No affinity[c]	No affinity[c]	No inhibition[c]	0.460 ± 0.24
3h	(4-BrC_6H_4)CH_2S–	No affinity[c]	No affinity[c]	No inhibition[c]	No inhibition[c]
3i	(4-$OCH_3C_6H_4$)CH_2S–	1.73 ± 0.45	No affinity[c]	No inhibition[c]	No inhibition[c]

[a] Values are expressed as the mean ± SEM of duplicate determinations.
[b] Values are expressed as the mean ± standard deviation (SD) of triplicate determinations.
[c] No affinity/inhibition observed at a maximum concentration of 100 μM of the test compound.

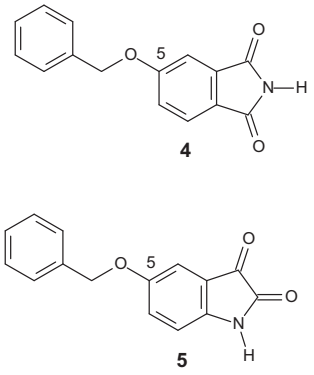


Fig. 5. The structures of 5-benzyloxyphthalimide (**4**) and 5-benzyloxyisatin (**5**).

Table 3
The K_i values for the binding of 5-benzyloxyphthalimide (**4**) and 5-benzyloxyisatin (**5**) to rat adenosine A_1 and A_{2A} receptors, and the reported IC_{50} values for the inhibition of human MAO-A and MAO-B.

	K_i (μM)[a]		IC_{50} (μM)	
	A_1	A_{2A}	MAO-A	MAO-B
4	4.67 ± 1.20	No affinity[b]	4.17[c]	0.043[c]
5	9.79 ± 2.07	No affinity[b]	4.62[d]	0.103[d]

[a] Values are expressed as the mean ± SEM of duplicate determinations.
[b] No affinity observed at a maximum concentration of 100 μM of the test compound.
[c] Values obtained from Ref. [24].
[d] Values obtained from Ref. [25].

promising candidate for dual action at adenosine A_1 receptors and MAO-B. Although exhibiting weaker affinities at adenosine A_1 receptors, **2a** [$K_i(A_1)$ = 0.648 μM], **2b** [$K_i(A_1)$ = 0.595 μM] and **2h** [$K_i(A_1)$ = 0.676 μM] are also examples of compounds with potential dual action at adenosine A_1 receptors and MAO-B. Compound **2h** also is a potent inhibitor of MAO-A (IC_{50} = 0.273 μM), a property which may be of value in the treatment of PD-associated depression. As discussed above, compound **3b** exhibits relatively good affinities for both adenosine A_1 receptors (K_i = 0.548 μM) and A_{2A} receptors (K_i = 0.980 μM). Compound **3b** was, however, a relatively weaker MAO-B inhibitor (IC_{50} = 1.88 μM), which makes it less suitable as a compound acting at both adenosine receptors and MAO-B.

3. Conclusion

The aim of the present study was to examine the possibility of developing dual-target-directed drugs that act as antagonists of adenosine receptors and inhibitors of MAO-B. Such compounds may act synergistic and offer enhanced benefit in the treatment of neurological diseases such as PD and AD. PD treatment: antagonism of the adenosine A_{2A} and A_1 receptors facilitate dopamine receptor signalling and dopamine release, respectively, while MAO-B inhibition may reduce the central catabolism of dopamine. AD treatment: antagonism of the adenosine A_1 receptor may be beneficial for the cognitive dysfuntion, while antagonism of the adenosine A_{2A} receptor and MAO-B inhibition display neuroprotective effects. Compound **2i** represents the most promising dual-target-directed compound identified in this study. Although **2i** possesses no affinity for adenosine A_{2A} receptors, it exhibits the highest affinity for the A_1 receptor among the compounds evaluated, and also is a highly potent MAO-B inhibitor. This compound may thus be considered as a promising candidate for dual action at adenosine A_1 receptors and MAO-B. Compound **2h** also is a noteworthy dual-target-directed compound since it is a potent inhibitor of both MAO-A and MAO-B, while also possessing good affinity for adenosine A_1 receptors. Although a limited number of derivatives were examined, and the adenosine receptor affinities may be considered as modest, this study provides "proof of concept" for the proposal that sulfanylphthalimide analogues may serve as promising leads for the design of dual acting adenosine antagonists and MAO inhibitors. This study also suggests that compounds that are structurally similar to the sulfanylphthalimides may represent leads for the design of dual-target-directed compounds. In this respect, a preliminary survey reveals that 5-benzyloxyphthalimide (**4**) [24] and 5-benzyloxyisatin (**5**) [25] (Fig. 5), compounds known to inhibit MAO-B, also possess modest affinities for adenosine A_1 receptors (Table 3). Compounds **4** and **5** exhibit K_i values of 4.67 μM and 9.79 μM, respectively for binding to adenosine A_1 receptors, while inhibiting MAO-B with IC_{50} values of 0.043 μM and 0.103 μM. In future studies, further structural optimization of these lead compounds may yield dual-target-directed compounds with improved activities.

4. Experimental section

4.1. Chemicals and instrumentation

Unless otherwise noted, all the reagents were obtained from Sigma–Aldrich and were used without further purification. Proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Ultrashield Plus 600 Avance III 600 spectrometer in DMSO-*d6* (Merck). The chemical shifts are reported in parts per million (δ) relative to the residual solvent signals. Spin multiplicities are abbreviated as follows: s (singlet), d (doublet), dd (doublet of doublets), t (triplet) or m (multiplet). High resolution mass spectra (HRMS) were recorded on a Bruker micrOTOF-Q II mass spectrometer in atmospheric-pressure chemical ionization (APCI) mode. Melting points (mp) were measured with a Buchi M-545 melting point apparatus and are uncorrected. Single-crystal X-ray diffraction analysis was carried out with a Bruker SMART X2S diffractometer. 5-Benzyloxyphthalimide (**4**) [24] and 5-benzyloxyisatin (**5**) [25] were synthesized as described in literature.

Counting of radio activity was performed using a Packard Tri-Carb 2100 TR liquid scintillation counter. [^{3}H]NECA (specific activity 25 Ci/mmol) and [^{3}H]DPCPX (specific activity 120 Ci/mmol) were obtained from Amersham Biosciences and PerkinElmer, respectively. Adenosine deaminase (type X from calf spleen; 250 units) and CPA were from Sigma–Aldrich. Whatman® GF/B 25 mm diameter filters and dimethyl sulfoxide (DMSO) were obtained from Merck. Filter-count was purchased from PerkinElmer. Fluorescence spectrophotometry was carried out with a Varian Cary Eclipse fluorescence spectrophotometer. Microsomes from insect cells containing recombinant human MAO-A and MAO-B (5 mg/mL) were from BD Biosciences while kynuramine.2HBr was obtained from Sigma–Aldrich.

4.2. Radioligand binding studies

The collection of tissue samples for the adenosine A_{2A} and A_1 receptor binding studies was approved by the Research Ethics Committee of the North-West University (application number NWU-0035-10-A5). The adenosine A_{2A} and A_1 receptor binding studies were carried out according the protocol described in literature [15,20]. For studies with the A_{2A} receptor, the dissected striata of male Sprague–Dawley rats were used while for the studies with the A_1 receptor whole rat brain (excluding cerebellum and brain stem) were employed. After dissection, the tissues were snap frozen with liquid nitrogen and stored at −70 °C. The tissue were thawed on ice, weighed and disrupted for 30 s (striata) or 90 s (whole brain) with the aid of a Polytron homogenizer (model: Polytron PT 10-35 GT) in 10 volumes of ice-cold 50 mM Tris buffer (pH 7.7 at 25 °C). The resulting homogenate was centrifuged at 20,000*g* for 10 min at 4 °C and the pellet was resuspended in 10 volumes of ice-cold Tris buffer, again with the aid of a Polytron homogenizer as above. The resulting suspension was recentrifuged and the pellet obtained was suspended in Tris buffer (pH 7.7 at 25 °C) to a volume of 5 mL/g original tissue weight. The striatal and whole brain membranes were aliquoted into microcentrifuge tubes and stored at −70 °C until needed. The incubations were carried out in 4 mL polypropylene tubes that were precoated with Sigmacote® (Sigma–Aldrich). All incubations were prepared with Tris buffer (pH 7.7 at 25 °C) to a volume of 1 mL. For the A_{2A} receptor binding study, the incubations contained 10 mg of the original tissue weight of the striatal membranes, 4 nM [^{3}H]NECA, 50 nM CPA, 10 mM $MgCl_2$, 0.2 units/mL adenosine deaminase, the test compound and 1% DMSO. DMSO was used to prepare all stock solutions of the compounds to be tested. For the A_1 receptor binding study, the incubations contained 5 mg of the original tissue weight of the whole brain membranes, 0.1 nM [^{3}H]DPCPX, 0.2 units/mL adenosine deaminase, the test compound and 1% DMSO. The incubations were vortexed and incubated for 60 min at 25 °C in a shaking waterbath. Half an hour after incubation was started, the incubations were vortexed again. The incubations were terminated via filtration through a prewetted 2.5 cm Whatman glass microfiber filter (grade GF/B) under reduced pressure using a Hoffeler vacuum system. The tubes were washed twice with 4 mL ice-cold Tris buffer and the filters were washed once more with 4 mL ice-cold Tris buffer. The damp filters were place in scintillation vials and 4 mL of scintillation fluid (Filter-Count) was added. The vials were shaken and incubated for two hours before being counted (Packard Tri-CARB 2100 TR). An estimate of the nonspecific binding was obtained from binding studies in the presence of 100 μM CPA. The IC_{50} values were determined by plotting the count values (adjusted for nonspecific binding) vs. the logarithm of the inhibitor concentrations to obtain a sigmoidal dose–response curve. These kinetic data were fitted to the one site competition model incorporated into the Prism software package (GraphPad Software Inc.). The K_i values for the competitive inhibition of [^{3}H]NECA (K_d = 15.3 nM) [20] or [^{3}H]DPCPX (K_d = 0.36 nM) [26] binding by the test compounds were calculated from the IC_{50} values according to the Cheng and Prusoff equation [27]. All incubations were carried out in duplicate and the K_i values are expressed as mean ± standard deviation (SD).

4.3. MAO inhibition studies

IC_{50} values for the inhibition of MAO-A and MAO-B were determined using the recombinant human enzymes as described previously [22,28]. The enzymatic reactions were carried out at pH 7.4 (K_2HPO_4/KH_2PO_4 100 mM, made isotonic with KCl) to a final volume of 500 μL. The reactions contained the different inhibitor concentrations spanning at least 3 orders of magnitude and the MAO-A/B mixed substrate kynuramine (45 μM for MAO-A and 30 μM for MAO-B). DMSO, as co-solvent (4%) was added to each reaction. The enzyme reactions were initiated with the addition of MAO-A or MAO-B (0.0075 mg protein/mL) and incubated for 20 min at 37 °C in a waterbath. After termination with the addition of 400 μL NaOH (2 N) and 1000 μL water, the concentrations of the MAO generated 4-hydroxyquinoline were measured by fluorescence spectrophotometry (λ_{ex} = 310; λ_{em} = 400 nM) [18]. For this purpose, a linear calibration curve containing authentic 4-hydroxyquinoline (0.047–1.56 μM) was constructed. The enzyme catalytic rates were calculated and fitted to the one site competition model incorporated into the Prism 5 software package (GraphPad). The IC_{50} values were determined in triplicate and are expressed as mean ± SD.

4.4. Single-crystal X-ray diffraction analysis

X-ray diffraction data were collected on a Bruker SMART X2S diffractometer using monochromated (doubly curved silicon crystal) Mo-Kα-radiation (0.71073 Å) and employing ω scan mode. For this purpose, a yellow coloured crystal of compound **3i** was mounted on a Mitegen Micromount with a small amount of epoxy and was automatically centred. Bruker APEX2 software was used for preliminary determination of the unit cell, and the determination of integrated intensities and unit cell refinement were performed with Bruker SAINT system. The data were corrected for absorption effects with SADABS using the multiscan technique. The structure was solved with XS and subsequent structure refinements were performed with XL [29]. The structure was refined by anisotropic full-matrix least-squares refinement on F^2: $C_{16}H_{13}NO_3S$, M = 299.34 g mol^{-1}, crystal size: 0.16 × 0.16 × 0.64 mm^3, monoclinic,

space group *P1 21/c1*, $a = 12.711(5)$ Å, $b = 5.3605(18)$ Å, $c = 20.449(8)$ Å, $\alpha = \gamma = 90$ °, $\beta = 100.434(13)$ °, $V = 1373.7(9)$ Å^3, $Z = 4$, $\rho_{calcd} = 1.447$ Mg cm^{-3}, $\mu = 0.245$ mm^{-1}, $\lambda = 0.71073$ Å, $T = 200(2)$ K, $\theta_{range} = 1.63–25.07$ °, reflections collected: 8049, independent: 2410 ($R_{int} = 0.0372$), 191 parameters, final R indices [$I > 2\sigma(I)$]: $R_1 = 0.0361$; $wR_2 = 0.0990$, max/min residual electron density: 0.208/−0.232 e$^-$/Å^3, goodness-of-fit on F^2 1.044, $F(000) = 624$. CCDC 1015897 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk.

4.5. Synthesis of the 4-sulfanylphthalimide analogues (3a–i)

A mixture of the appropriate thiol (6 mmol), 3-nitrophthalimide (4 mmol) and K_2CO_3 (10 mmol) in 20 mL acetone was heated under reflux for 24 h. The reaction was cooled to room temperature and diluted with 150 mL water. The mixture was subsequently acidified to pH 2 with 6 N HCl. The resulting precipitate was collected by filtration and dried at 50 °C [19].

4.5.1. 4-(Phenylsulfanyl)phthalimide (**3a**)

The title compound (yellow crystals) was prepared from 3-nitrophthalimide and thiophenol in a yield of 62%: mp 209.0–210.4 °C (ethanol); **^{1}H NMR** (Bruker Avance III 600, DMSO-*d6*) δ 6.85 (d, 1H, J = 7.9 Hz), 7.51 (d, 1H, J = 7.2 Hz), 7.54–7.58 (m, 4H), 7.60–7.62 (m, 2H), 11.40 (s, 1H); **^{13}C NMR** (Bruker Avance III 600, DMSO-*d6*) δ 119.3, 126.3, 128.5, 130.1, 130.1, 130.4, 133.8, 134.7, 135.5, 138.2, 168.7, 168.7; **APCI-HRMS** *m/z*: calcd for $C_{14}H_{10}NO_2S$ $[MH]^+$, 256.0432, found 256.0426; **Purity** (HPLC): 100%.

4.5.2. 4-(Benzylsulfanyl)phthalimide (**3b**)

The title compound (light yellow needles) was prepared from 3-nitrophthalimide and benzyl mercaptan in a yield of 30%: mp 211.5–213.5 °C (ethanol); **^{1}H NMR** (Bruker Avance III 600, DMSO-*d6*) δ 4.39 (s, 2H), 7.25–7.28 (m, 1H), 7.32–7.35 (m, 2H), 7.46 (d, 2H, J = 7.5 Hz), 7.51 (d, 1H, J = 6.8 Hz), 7.67–7.73 (m, 2H), 11.30 (s, 1H); **^{13}C NMR** (Bruker Avance III 600, DMSO-*d6*) δ 34.0, 118.6, 126.7, 127.4, 128.6, 129.0, 130.1, 133.8, 134.5, 136.2, 137.8, 168.7, 168.9; **APCI-HRMS** *m/z*: calcd for $C_{15}H_{12}NO_2S$ $[MH]^+$, 270.0589, found 270.0557; **Purity** (HPLC): 100%.

4.5.3. 4-[(2-Phenylethyl)sulfanyl]phthalimide (**3c**)

The title compound (white needles) was prepared from 3-nitrophthalimide and 2-phenylethyl mercaptan in a yield of 31%: mp 150.2–151.6 °C (ethanol); **^{1}H NMR** (Bruker Avance III 600, DMSO-*d6*) δ 2.96 (t, 2H, J = 7.6 Hz), 3.35 (t, 2H, J = 7.6 Hz), 7.20–7.23 (m, 1H), 7.28–7.32 (m, 4H), 7.51 (dd, 1H, J = 1.5, 7.9 Hz), 7.69–7.72 (m, 2H), 11.28 (s, 1H); **^{13}C NMR** (Bruker Avance III 600, DMSO-*d6*) δ 30.9, 33.9, 118.4, 126.4, 126.8, 128.4, 128.6, 129.8, 133.9, 134.6, 137.8, 139.7, 168.7, 168.8; **APCI-HRMS** *m/z*: calcd for $C_{16}H_{14}NO_2S$ $[MH]^+$, 284.0745, found 284.0744; **Purity** (HPLC): 100%.

4.5.4. 4-[(4-Chlorophenyl)sulfanyl]phthalimide (**3d**)

The title compound (yellow crystals) was prepared from 3-nitrophthalimide and 4-chlorothiophenol in a yield of 53%: mp 268.9–271.0 °C (ethanol/ethyl acetate); **^{1}H NMR** (Bruker Avance III 600, DMSO-*d6*) δ 6.92 (d, 1H, J = 7.9 Hz), 7.54 (d, 1H, J = 7.2 Hz), 7.58–7.63 (m, 5H), 11.41 (s, 1H); **^{13}C NMR** (Bruker Avance III 600, DMSO-*d6*) δ 119.6, 126.6, 127.8, 130.4, 130.5, 133.8, 134.9, 135.1, 137.0, 137.4, 168.7, 168.7; **APCI-HRMS** *m/z*: calcd for $C_{14}H_9ClNO_2S$ $[MH]^+$, 290.0043, found 290.0037; **Purity** (HPLC): 100%.

4.5.5. 4-[(4-Bromophenyl)sulfanyl]phthalimide (**3e**)

The title compound (yellow crystals) was prepared from 3-nitrophthalimide and 4-bromothiophenol in a yield of 45%: mp 270.3–271.9 °C (ethanol); **^{1}H NMR** (Bruker Avance III 600, DMSO-*d6*) δ 6.93 (d, 1H, J = 7.9 Hz), 7.53–7.55 (m, 3H), 7.59–7.61 (m, 1H), 7.73 (d, 2H, J = 8.3 Hz), 11.40 (s, 1H); **^{13}C NMR** (Bruker Avance III 600, DMSO-*d6*) δ 119.7, 123.8, 126.7, 128.4, 130.6, 133.3, 133.8, 134.9, 137.1, 137.1, 168.7, 168.7; **APCI-HRMS** *m/z*: calcd for $C_{14}H_9BrNO_2S$ $[MH]^+$, 333.9537, found 333.9535; **Purity** (HPLC): 100%.

4.5.6. 4-[(4-Fluorobenzyl)sulfanyl]phthalimide (**3f**)

The title compound (yellow needles) was prepared from 3-nitrophthalimide and 4-fluorobenzyl mercaptan in a yield of 70%: mp 179.8–180.2 °C (ethanol/ethyl acetate); **^{1}H NMR** (Bruker Avance III 600, DMSO-*d6*) δ 4.36 (s, 2H), 7.15 (t, 2H, J = 8.7 Hz), 7.47–7.51 (m, 3H), 7.66–7.70 (m, 2H), 11.30 (s, 1H); **^{13}C NMR** (Bruker Avance III 600, DMSO-*d6*) δ 33.4, 115.5, 115.6, 118.9, 126.8, 130.3, 131.2, 131.2, 132.5, 133.9, 134.7, 137.7, 160.8, 162.4, 168.9, 169.0; **APCI-HRMS** *m/z*: calcd for $C_{15}H_{11}FNO_2S$ $[MH]^+$, 288.0489, found 288.0495; **Purity** (HPLC): 100%.

4.5.7. 4-[(4-Chlorobenzyl)sulfanyl]phthalimide (**3g**)

The title compound (yellow crystals) was prepared from 3-nitrophthalimide and 4-chlorobenzyl mercaptan in a yield of 61%: mp 233.5–239.4 °C (ethanol/ethyl acetate); **^{1}H NMR** (Bruker Avance III 600, DMSO-*d6*) δ 4.37 (s, 2H), 7.37 (d, 2H, J = 8.3 Hz), 7.47 (d, 2H, J = 8.7 Hz), 7.49–7.51 (m, 1H), 7.66–7.67 (m, 2H), 11.30 (s, 1H); **^{13}C NMR** (Bruker Avance III 600, DMSO-*d6*) δ 33.4, 119.0, 126.9, 128.7, 130.3, 131.0, 132.2, 133.9, 134.7, 135.6, 137.5, 168.9, 169.0; **APCI-HRMS** *m/z*: calcd for $C_{15}H_{11}ClNO_2S$ $[MH]^+$, 304.0199, found 304.0099; **Purity** (HPLC): 100%.

4.5.8. 4-[(4-Bromobenzyl)sulfanyl]phthalimide (**3h**)

The title compound (light yellow crystals) was prepared from 3-nitrophthalimide and 4-bromobenzyl mercaptan in a yield of 65%: mp 261.3–262.1 °C (ethanol/ethyl acetate); **^{1}H NMR** (Bruker Avance III 600, DMSO-*d6*) δ 4.38 (s, 2H), 7.42 (d, 2H, J = 8.7 Hz), 7.51–7.53 (m, 3H), 7.67–7.70 (m, 2H), 11.31 (s, 1H); **^{13}C NMR** (Bruker Avance III 600, DMSO-*d6*) δ 33.2, 118.8, 120.5, 126.8, 130.2, 131.2, 131.5, 133.8, 134.5, 135.9, 137.3, 168.6, 168.8; **APCI-HRMS** *m/z*: calcd for $C_{15}H_{11}BrNO_2S$ $[MH]^+$, 347.9694, found 347.9591; **Purity** (HPLC): 100%.

4.5.9. 4-[(4-Methoxybenzyl)sulfanyl]phthalimide (**3i**)

The title compound (yellow crystals) was prepared from 3-nitrophthalimide and 4-methoxybenzyl mercaptan in a yield of 44%: mp 220.7–223.7 °C (ethanol); **^{1}H NMR** (Bruker Avance III 600, DMSO-*d6*) δ 3.71 (s, 3H), 4.30 (s, 2H), 6.88 (d, 2H, J = 8.3 Hz), 7.36 (d, 2H, J = 8.7 Hz), 7.49 (d, 1H, J = 6.8 Hz), 7.66–7.71 (m, 2H), 11.31 (s, 1H); **^{13}C NMR** (Bruker Avance III 600, DMSO-*d6*) δ 33.7, 55.2, 114.2, 118.8, 126.7, 127.9, 130.3, 130.4, 133.9, 134.7, 138.2, 158.7, 169.0, 169.0; **APCI-HRMS** *m/z*: calcd for $C_{16}H_{12}NO_3S$ $[M-H]^+$, 298.0538, found 298.0538; **Purity** (HPLC): 100%.

4.6. HPLC traces of the 4-sulfanylphthalimide analogues (3a–i)

The degree of purity for each of the synthesized 4-sulfanylphthalimide analogues (**3a-i**) were estimated with HPLC analyses, which were carried out with an Agilent 1100 HPLC system equipped with a quaternary pump and an Agilent 1100 series diode array detector. Milli-Q water (Millipore) and HPLC grade acetonitrile (Merck) were used for the chromatography of compounds **3a**, **3b**, **3c** and **3f**, and HPLC grade tetrahydrofuran (Sigma–Aldrich) were used for the chromatography of compounds **3d**, **3e**, **3g**, **3h** and **3i**. A Venusil XBP C18 column (4.60 × 150 mm, 5 μm) was used and the mobile phase consisted initially of 30% acetonitrile

or tetrahydrofuran and 70% MilliQ water at a flow rate of 1 mL/min. At the start of each HPLC run a solvent gradient program was initiated by linearly increasing the composition of the acetonitrile or tetrahydrofuran in the mobile phase to 85% acetonitrile or tetrahydrofuran over a period of 5 min. Each HPLC run lasted 15 min and a time period of 5 min was allowed for equilibration between runs. A volume of 20 μL of solutions of the test compounds in acetonitrile or tetrahydrofuran (1 mM) was injected into the HPLC system and the eluent was monitored at wavelengths of 254 nm.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2015.02.005.

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